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Supercritical CO_2 extraction of carotenoids and other lipids from *Chlorella vulgaris*

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Freeze-dried samples of the microalga *Chlorella vulgaris* were submitted to supercritical CO_2 at temperatures of 40 and 55°C and pressures up to 35·0 MPa. This study was carried out on whole and crushed algae. The extraction yields of carotenoids and other lipids were low in the former case and improved significantly in the latter one. Extraction yields of carotenoids and other lipids increased with pressure. The fraction of carotenoids in the oil was also greater at higher pressures. On the other hand, supercritical CO_2 extraction of carotenoids compared favourably with hexane and acetone extractions.

INTRODUCTION

Microalgae can be an important source of carotenoids for commercial use, since these compounds can be obtained in high yield (Borowitzka, 1988). *Chlorella vulgaris*, grown at the Departamento de Energias Renováveis — INETI, has produced carotenoids (mainly canthaxanthin and astaxanthin) in fairly high yields (Novais *et al.*, 1993).

Among the naturally occurring carotenoids only three are at present produced synthetically: β-carotene, canthaxanthin and astaxanthin. β-Carotene has an activity of provitamin A and is a preventive of cancer (Anon., 1993; Mordi, 1993), while others, like canthaxanthin, though without that activity, can also enhance the immune response to this disease (Bendich, 1991). Canthaxanthin and astaxanthin have been used as pigments in feeds to colour salmonid fish, trout and egg yolk and also in the food industries. The market for natural carotenoids from algae, plants and microbial sources is restricted, but has been growing fast (Johnson & Hwan, 1991; Anon., 1992; Iwasaki & Murskoshi, 1992).

Carotenoids extracted by supercritical CO_2 will be free of potential harmful organic solvents. Solubility measurements of β-carotene in that solvent have been carried out (Cygnarowicz *et al.*, 1990; Sakaki, 1992), as well as supercritical fluid extraction (SFE) of this compound from plants and algae (Stahl *et al.*, 1988; Erazo *et al.*, 1989), of astaxanthin and its esters from Antarctic krill (Yamaguchi *et al.*, 1986) and of bixin from Annato seed (Chao *et al.*, 1991; Degnan *et al.*, 1991).

Supercritical carbon dioxide extractions with microalgae have been carried out, namely to obtain hydrocarbons from *Botryococcus braunii* (Mendes *et al.*, 1994) and lipids from *Ochromonas danica* and *Skeletonema costatum* (Polak *et al.*, 1989).

The objective of this work was to assess the feasibility of extracting, with supercritical CO_2, carotenoids and other lipids from *Chlorella vulgaris*.

MATERIALS AND METHODS

Supercritical fluid extraction apparatus

A flow type apparatus, which allowed to carry out studies of supercritical fluid extraction at pressures and temperatures up to 40·0 MPa and to 60°C, respectively (Mendes *et al.*, 1995), was built (Fig. 1).

The liquid CO_2 (Air liquid, 99·995% purity) is compressed in the extraction vessels (extractors) 9 and 10, through the pump, 4, the pressure being controlled by the back-pressure regulator, 5, and measured by a Bourdon-type gauge, 8 (0–50·0 MPa). Before reaching the extractors, the fluid passed through a heat exchanger, 7. The extractors and the heat exchanger were immersed in a controlled temperature water bath.

In this work, only one of the extraction vessels was used, in which 5 g of algae were introduced to occupy about one-third of the extractor. Its lower part was filled with glass wool, which was also placed at the top to avoid the entrainment of the material.

After depressurisation, the lipids were recovered in glass U-tubes, 12, immersed in a ice bath. These tubes

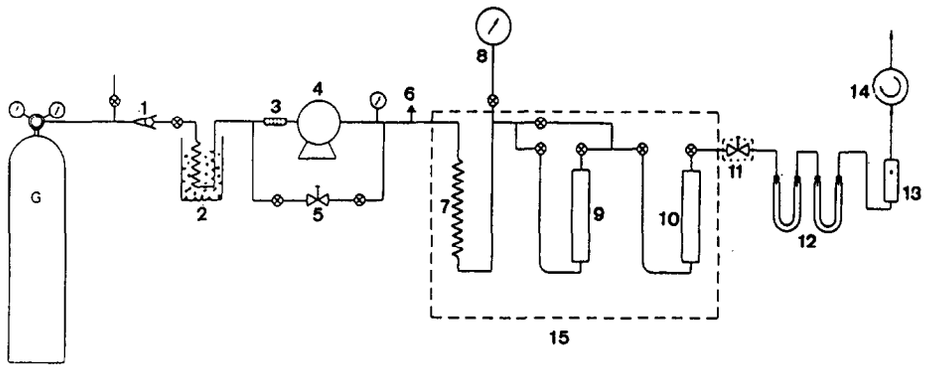

Fig. 1. Diagram of the supercritical fluid extraction apparatus. 1, Check valve; 2, ice cooler; 3, filter; 4, pump; 5, back-pressure regulator; 6, rupture disc; 7, heat exchanger; 8, manometer; 9 and 10, extraction vessels; 11, expansion valve; 12, glass U-tubes (collectors); 13, rotameter; 14, wet test meter; and 15, water bath.

were weighed before and after the extraction. The CO_2 flow-rate was monitored with the rotameter, 13, and the total volume was measured with the wet test meter, 14.

Total lipids and solvent extraction

About 150 mg of algae were used to allow the extraction and analysis of total lipids, with methanol, chloroform and water, by the method of Bligh and Dyer (1959).

Also *n*-hexane and acetone extractions were carried out on two samples of 400 mg of crushed algae. These extractions were accomplished during 72 h, by adding to algae, with stirring, 150 ml of solvent, in three steps of 50 ml each.

Microalga culture

The starting strain of *Chlorella vulgaris* was obtained from a contaminant of an open-air culture of the microalga *Botryoccocus braunii* UC 058. The culture was optimised and the induction of carotenogenesis was achieved through saline, luminous or nutritional stress, just before the stationary phase (Novais *et al.*, 1993). The orange alga was freeze-dried and maintained at –20°C in a nitrogen atmosphere for further utilisation.

Fatty acids analysis

For analysis of the fatty acids in algae and in supercritical CO_2 extracts, the method of Lepage and Roy (1986), modified by Cohen *et al.* (1988), was used to obtain the methylic esters. These were analysed by gas chromatography (GC), in a 'Varian 3300' chromatograph with a flame ionisation detector and a capillary column of fused silica, Supelcowax 10 (Supelco), 30 m × 0·32 mm (i.d.). The carrier gas was helium with a split ratio of 100 and a flow rate of 1·5 ml min^{-1}. The working temperatures were: 200, 300 and 175°C at the injector, detector and column, respectively. The column temperature was maintained for 5 min at this value, then increased up to 235°C (2·5°C min^{-1}) and kept at this value for 15 min. The fatty acids were identified by comparing the retention times with those of mixtures of known composition (sardine and cod liver oils) (Morris & Culkin, 1976). The oils were obtained and prepared from fresh sardines and cod liver capsules, in darkness and nitrogen atmosphere, and an antioxidant, 2,6-di-*tert*-butyl-4-methylphenol, was added (0·01% w/w) to them. Heptadecanoic acid was used as internal standard (Morris & Culkin, 1976).

Thin-layer chromatography of carotenoids

The carotenoids from algae were extracted with acetone after breaking the cell walls through mechanical and thermic shocks (about 100 mg of algae with glass beads are put alternately in an ice bath and in a vortex). The separation of carotenoids, for further identification and quantification, was carried out by thin-layer chromatography (TLC) using silica gel plates with 0·25 mm thickness (Merck), activated for 1 h at 120°C and an eluent mixture of petroleum-ether (80–100°C)/acetone/diethylamine (10 : 4 : 1, v/v/v). The identification of carotenoids was achieved by colour, R_f values and wavelength at maximum absorbance (Reis, 1992). The quantification was made by spectrophotometry, (Spectrophotometer Philips-PU 8620 UV/Vis/NIR).

Total pigments

A quicker method was also used to assess the total carotenoids from the alga samples and the supercritical CO_2 extracts. The total pigments were determined in terms of canthaxanthin equivalent, i.e. by measuring the absorbance of the dissolved extracts in acetone at 466 nm. Hence, using an experimental value of the optical extinction coefficient (E = 2109) (Reis, 1992) and a light path of 1 cm the correlation $M = 4{\cdot}74\ A$ was obtained, where M is the concentration of carotenoids in acetone (mg $litre^{-1}$) and A is the measured absorbance.

Canthaxanthin and astaxanthin (HPLC)

Astaxanthin and canthaxanthin from supercritical CO_2 extracts were quantified by high-performance liquid chromatography (HPLC). The system consisted of a

liquid chromatograph Perkin–Elmer Series 10 with a UV/Vis detector LC-25 at 460 nm, coupled to a Perkin–Elmer LCI-100 integrating unit, and a reversed phase 10 μ C-18 (Waters) 3·9 × 300 mm column. As eluent, a mixture of acetonitrile/methanol/water (75 : 25 : 10, v/v/v) with a flow rate of 1·0 ml min^{-1} was used. Calibration curves, for the analysis of the extracts, were obtained using canthaxanthin (Fluka) and astaxanthin (Roche) solutions in acetone, whose concentrations were previously determined by spectrophotometry.

RESULTS AND DISCUSSION

Composition of the alga

Microalgae used in these studies showed the following composition per 100 g of dry weight: 24·5 g of lipids (Bligh & Dyer method), 133 mg of carotenoids (TLC) and 12·1 g of fatty acids (GC after transmethylation). C18 : 1, C16 : 0 and C18 : 3 were found to be the main constituents of the glycerides and fatty acids fraction, with 41, 22 and 9% weight, respectively.

Supercritical fluid extraction of algae (whole cells)

Figure 2 shows SFE curves at two pressures (20·0 and 35·0 MPa) and two temperatures (40°C and 55°C). It was found that the oil extraction yield increased either with pressure at constant temperature or with temperature at constant pressure. The extraction curves consist of two zones: one that corresponds to the solubility of outer lipids of the cells and another where the extraction seems to be controlled by the diffusion within the cells.

SFE curves of carotenoids are shown in Fig. 3. The extraction yield of these compounds at 55°C increased greatly with pressure, as well as fractions in the extracts (Table 1).

The carotenoid fractions also increased in the course of the extraction. For example, at 35·0 MPa (55°C), the following fractions were obtained: 143 mg/100 g of extracted oil at the first collected extract, 158 mg at the second, 211 mg at the third, 547 mg at the fourth. The extracts that initially were fluid and with orange colour became dark red and viscous.

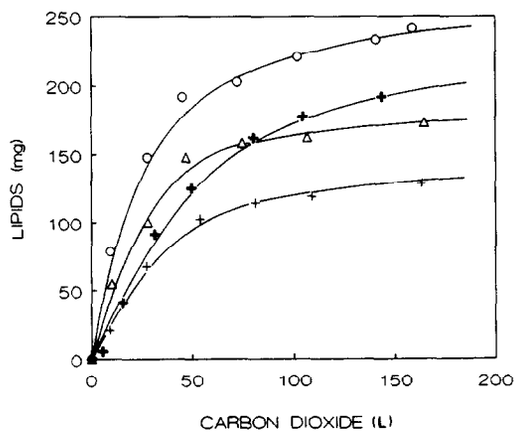

Fig. 2. Extracted lipids from whole algae (5 g) as a function of CO_2 volume (STP). Gas flow rate, 0·4 litres min^{-1}. Particle diameter, 0·4 mm (+) 20·0 MPa/40°C; (✚) 20·0 MPa/55°C; (Δ) 35·0 MPa/40°C; (○) 35·0 MPa/55°C.

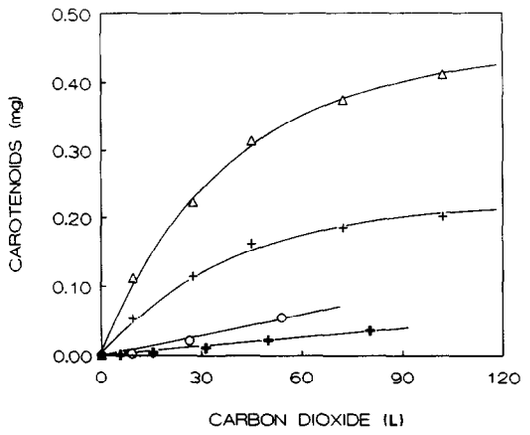

Fig. 3. Extracted carotenoids and canthaxanthin as a function of CO_2 volume (STP). (○) 20·0 MPa/40°C; (✚) 20·0 MPa/55°C; (Δ) 35·0 MPa/55°C; (+) canthaxanthin, 35·0 MPa/55°C.

Table 1. Fraction of carotenoids (mg/100 g (oil)) at T = 55°C and 50 litres gas (STP)

Pressure (MPa)	Fraction
15·0	11·3
20·0	17·6
27·5	138·8
35·0	171·1

The alga residues after supercritical extraction present different colorations according to the attained degree of extraction. These residues are more yellow and less red when they suffer a more intense extraction. The extracts are more viscous (at the plateau zone of the extraction curves), because there was a reduction of the fatty acid fraction and, therefore, an increase of the wax fraction: 48% wt of fatty acids (glycerides) in the first collected extract at 35·0 MPa (55°C), while in the fifth that fraction dropped to 39%.

Canthaxanthin is the major carotenoid component either in algae or in the supercritical extracts and the evolution of its extraction yield with CO_2 volume at 35·0 MPa/55°C is shown in Fig. 3. The solubility of carotenoids in supercritical CO_2 also depends on the length of the hydrocarbon chain and the presence of functional groups, as well as on the entrainment effect of the other lipids (fatty oils and waxes) (Stahl *et al.*, 1988).

Supercritical fluid extraction of algae (crushed cells)

Figure 4 shows SFE curves of crushed algae. Extraction yield, which was higher, for the same conditions, than the one obtained with whole cells, increased with pressure. At 20 MPa it decreased with temperature; however at 35 MPa the yield increased with temperature, but the effect was less pronounced.

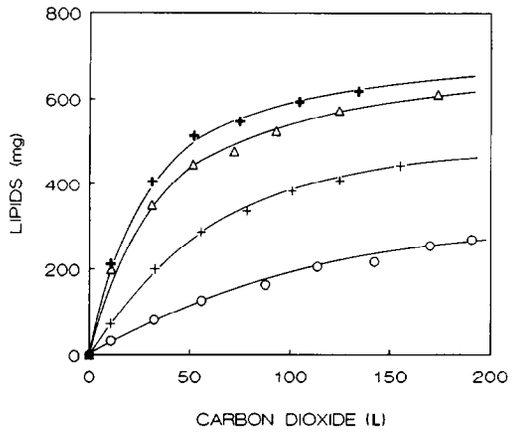

Fig. 4. Extracted lipids from crushed algae (5 g) as a function of CO_2 volume (STP). Gas flow rate 0·4 litres min^{-1}. (+) 20·0 MPa/40°C; (○) 20·0 MPa/55°C; (Δ) 35·0 MPa/40°C; (✚) 35·0 MPa/55°C.

The maximum yield of lipids obtained by supercritical extraction, based on dry weight of crushed algae, was 13·3%, at 35·0 MPa/55°C, while it was 5% with whole algae. However, the yield for acetone and hexane extractions gave a yield of 16·8 and 18·5%, respectively.

The residues, after SFE at 35·0 MPa, had, at the extractor bottom, a white-grey appearance (total extraction of carotenoids) and gradually became coloured across the bed.

Figure 5 shows extraction curves of carotenoids at several conditions of pressure and temperature. Extraction yield of these compounds increased with pressure. On the other hand, at 20 MPa it decreased with temperature, while at 35 MPa an increase was observed. The achieved yields by supercritical, hexane and acetone extraction are shown in Table 2. The yields obtained with organic solvents are lower than those reached with supercritical CO_2 at 35·0 MPa.

Figure 6 depicts the extraction of canthaxanthin and astaxanthin at 35·0 MPa/40°C. These compounds represent about two-thirds of the extracted carotenoids. Astaxanthin is more polar than canthaxanthin and this

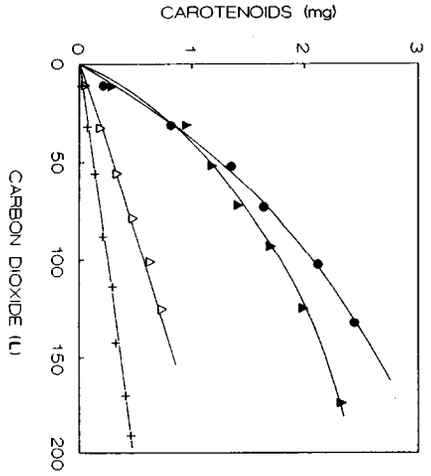

Fig. 5. Extracted carotenoids as a function of CO_2 volume (STP). (Δ) 20·0 MPa/40°C; (+) 20·0 MPa/55°C; (▲) 35·0 MPa/40°C; (●) 35·0 MPa/55°C.

Table 2. Supercritical carbon dioxide extraction yield of carotenoids from microalga *Chlorella vulgaris* (crushed cells)

Pressure (MPa)/ Temperature (°C)	CO_2 volume (STP) (litres)	Yield (%, w/w (alga))
20·0/40	125	0·01
20·0/55	191	0·01
27·5/40	122	0·02
27·5/55	126	0·02
35·0/40	174	0·05
35·0/55	132	0·05
Hexane	—	0·03
Acetone	—	0·04

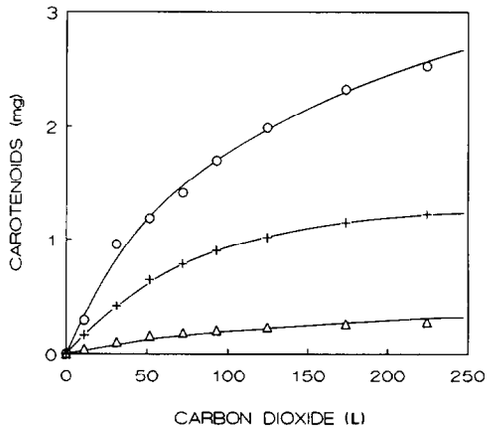

Fig. 6. Extracted canthaxanthin, astaxanthin and total carotenoids at 35·0 MPa/40°C as a function of CO_2 volume (STP). (Δ) Astaxanthin; (+) canthaxanthin; (○) total carotenoids.

implies a lower extraction yield of that compound in supercritical CO_2.

CONCLUSIONS

Higher pressures led to a higher efficiency in the SFE of lipids and carotenoids from the crushed microalga, *Chlorella vulgaris*. A small increase of temperature, at higher pressures, led to a slightly higher extraction yield. At lower pressures the temperature had an opposite effect on the extraction yield of carotenoids and other lipids.

SFE of algae with whole cells led to low yields of carotenoids and lipids (5% dry weight at 35·0 MPa/55°C), while the use of disrupted cells led to higher yields (13·3% at the same conditions). At 35·0 MPa the supercritical extraction yield of carotenoids with crushed cells compared favourably with acetone and hexane extraction yields.

Varying the extraction pressure, and also the volume of CO_2 used, it was possible to modify the carotenoid fraction in the extracted oils. To increase the yield of extraction and at the same time to protect the carotenoids against degradation, the addition of vegetable oils, due to their entrainment effect, to the crushed algae is an interesting alternative.

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